The mycorrhizal associations of *Borya mirabilis*, an endangered Australian native plant

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Introduction

The genus *Borya* Labill. was described in 1805, based on the Western Australian species *B. nitida* (Labillardiere 1805). *Borya* was long included in the Liliaceae but was subsequently placed in the tribe Boryeae within the family Boryaceae (Baker 1879). Placement in the Boryaceae, with one other genus *Alania* Endl., has been confirmed by molecular studies (Chase *et al.* 1996). *Borya* species are considered desiccation tolerant and lose most of their chlorophyll and thylakoids when dehydrated and can survive the loss of up to 94% of their water (Porembski & Barthlott 2000).

Thirteen species of *Borya* have been described, three of which are threatened (Reiter 2008). The greatest diversity occurs in mediterranean south-western Western Australia (Reiter 2008) and in tropical Australia (Western Australia, Northern Territory and Queensland) with four species. Only *B. mirabilis* has a south-eastern Australian distribution. This species is confined to the Grampians (Gariwerd) National Park approximately 250 km north-west of Melbourne. Currently only one natural population of the species is known, in the Wonderland Range, with a second, translocated, population some 15 km north of this in the Mt Difficult Range. Despite close monitoring since its description (Churchill 1985), there has been no production of viable seed in *B. mirabilis* and the sole population is believed to comprise a single clone or plants that are so genetically similar that they are incapable of sexual reproduction (Coates *et al.* 2002). As a consequence it is one of Australia's most endangered plants.

Borya mirabilis is listed as endangered nationally under the *Environment Protection and Biodiversity Conservation Act 1999* and in Victoria is listed as a threatened taxon under the *Flora and Fauna Guarantee Act 1988*. An *ex-situ* population established at the Royal Botanic Gardens Melbourne (RBG) is of particular importance. A National Recovery Plan has been prepared (Coates *et al.* 2002) and a recovery team has been formed to

Abstract

The mycorrhizal associations of *Borya mirabilis* Churchill, T.B.Muir & Sinkora are described for the first time, based on seasonal observations over 12 months. Roots of vascular plants associated with *B. mirabilis* in the field were also examined for mycorrhizal associations; *Callitris rhomboidea* R.Br. ex Rich. & A.Rich. was found to be most morphologically similar. The usefulness of associated plants to inoculate *ex-situ* populations and as indicators of potential translocation sites for *B. mirabilis* is discussed.

Key words: Boryaceae, arbuscular mycorrhizae, propagation.

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implement the recovery plan, in particular, to increase the number of plants in cultivation and to establish self-sustaining translocated populations in the wild. However, management is hampered by a lack of basic knowledge about growth requirements, particularly in relation to mycorrhizal associations, media requirements and reproductive biology.

Mycorrhizal associations are of particular importance in the management of any plant species. No studies on the mycorrhizae of *Borya* species have been published; however, Keighery (1984) refers to Western Australian *Borya* species as being both ectomycorrhizal and arbuscular mycorrhizal.

The fungi that form arbuscular mycorrhizae (AM) are biotrophs. They provide a direct link between plant roots and the soil (Smith & Read 1997). Benefits of AM include increased phosphorous uptake (George *et al.* 1992; Titus & Lepš 2000); increased uptake of nitrogen, magnesium and iron (Fitter & Merryweather 1992; Barea & Jeffries 1995; Varma 1995), zinc and copper (Marschner 1995); increased resistance to stress, pathogens, heavy metals, acidity and salinity (Pfetffer & Bloss 1988; Dodd *et al.* 1990; George *et al.* 1992; Barea & Jeffries 1995; Marschner 1995); changing compounds in soil to easily absorbed forms (Leyval & Berthelin 1989; Knight *et al.* 1989; Marschner & Dell 1994); and general increased plant size and vigour (Grime and Hodgson 1987; Smith & Read 1997; Bohrer *et al.* 2003; van der Heijden *et al.* 2003). Ectomycorrhizae (EM) are symbiotic mycorrhiza, which differ from AM in having septate hyphae a Hartig net and extracellular mantle (Brundrett 2008). Similar benefits have been found with EM as in AM (Buscot *et al.* 2000).

Arbuscular mycorrhizae can be assigned to three morphological types: Arum type, Paris type, and a third type known as *Rhizophagus* or *Endogone* mycorrhizae. This third group of AM is associated with plants that form nodular structures on their roots (Saxton 1930; Béreau & Garbaye 1994). The following questions are addressed in this paper:

1. What type(s) of mycorrhizae does *Borya mirabilis* form and how does this alter seasonally?
2. Do the fungi provide a beneficial relationship to *Borya mirabilis* when growing together?
3. What mycorrhizal associations do associated plants in the field have?

Material and methods

Borya mirabilis field sampling

Root samples from *Borya mirabilis* were collected each season over a 12-month period from the one known field site. Three 50 g samples of roots and soil were collected from four different colonies each season. Samples were stored in 50% ethanol and taken back to the laboratory for clearing and staining. Root samples were gently rinsed in tap water and placed in 10% potassium hydroxide, warmed to 90°C for a period of two hours and left to cool and clear for 30 minutes. The roots were then rinsed twice in distilled water for five minutes, placed in warm lacto-fuchsin (Carmichael 1955) for five minutes and cleared in three changes of 60°C glycerol over several days and finally stored in 50% glycerol for examination (Brundrett 2008). Thirty root samples were randomly chosen from each processed 50 g sample. The roots where then observed under a light microscope. Root morphology, type of mycorrhizae present and infection percentage were recorded. Mycorrhizae were divided broadly into four groups: ectomycorrhiza; arbuscular mycorrhiza; ericoid mycorrhiza and orchid mycorrhiza. The division was based on characters that define these groups (Brundrett 2008) including: the presence or absence of septate hyphae; a sheath; hyphae inside plant cells and hyphal coils or arbuscules. The percent mycorrhizal infection of each of these roots was determined by determining the total root area using a micrometer, dividing this by the area infected with mycorrhizal fungi and multiplying by 100.

Mycorrhizal pot trials

Root samples of *B. mirabilis* were taken from the field site and used to inoculate three pots of *B. mirabilis* in the *ex-situ* population at the Royal Botanic Gardens Melbourne. Pots were left for three months to allow time for root infection. The roots and soil of these plants were then used to inoculate the *ex-situ* population, with 11 plants being inoculated and 11 left as controls. Plants were re-potted in sterile potting media, with a layer of glass beads over the surface to prevent infection of the soil by air-borne fungal spores. A sealed watering tube was inserted so plants could be watered with sterile water.

Potential infection by *Phytophthora cinnamomi* Rands was controlled in two ways. Firstly, field samples

were taken from *B. mirabilis* plants which appeared uninfected by *P. cinnamomi* and all field equipment and shoes were sprayed with Phytoclean on entering and exiting the site. Secondly, plants inoculated with field soil and roots were treated with furalaxyl and metalaxyl in order to kill any *Phytophthora* that may have been present in the soil.

Plants from both treatments were housed in a quarantine greenhouse at the Royal Botanic Gardens Melbourne and in all other respects were treated equally. Plants were monitored once a month for six months and the number of shoots and leaf colour were recorded. At the conclusion of the trial, root samples were taken. They were cleared, stained and viewed for the presence/absence of mycorrhizae, using the same methodology as for field sampled roots. The results were analysed by ANOVA using Minitab 14© software.

Associated species mycorrhizal examination

Vascular plant species associated with *B. mirabilis* in the field were previously identified during an ecological study conducted on the site (Reiter 2008), During winter, three root samples from each of three individual plants from 19 species associated with *B. mirabilis* in the field were taken. Several additional seasonal species and the other threatened plant species on the site were not examined due to low numbers of these species on the site. The associated mosses on the field site were not examined, as mycorrhizal associations have never been observed in mosses (Read et *al.* 2000; Davey & Currah 2006).

All associated plants that were sampled occurred within 10 m of *B. mirabilis* plants. Plant roots were stored in 50% ethanol, cleared, stained as with *B. mirabilis* (with the exception that tougher roots required longer clearing, depending on the species) and examined for the presence of mycorrhizae. Plants infected with ectomycorrhizae (EM) were embedded in wax and cross-sectioned to observe internal structures (Reiter 2008). Thirty slides from each associated species were examined and the type of mycorrhizal infection recorded. The mycorrhizal type was determined as AM, EM, ericoid or orchid, based on septation of the hyphae, presence of hyphal coils (absent in EM), presence or absence of arbuscules and vesicles (characteristic of AM) and presence or absence of a mantle and Hartig net (characteristic of EM), as well as the host family, following Harley and Smith (1983), Schüßler (2002) and Selosse *et al.* (2007).

Results

Borya mirabilis field sampling

The roots of *Borya mirabilis* contained small nodules along the lengths of the lateral roots, matching those described in 'Endogone-type' AM. These nodules had indeterminate growth and would often appear on top of each other as offshoots (Fig. 1).

Examination of roots over each season throughout the year showed clearly that the roots of *B. mirabilis* were predominantly associated with AM, but some EM were also present. The AM appeared as spores within the nodules on the roots in summer (Fig. 1), and as coils contained inside the nodule cells in winter (Fig. 1). The EM structures observed were predominantly of the *Cenococcum* type. Infection by AM was greater in winter and spring than in summer and autumn; while infection by EM was highest in autumn and winter (Table 1).

Mycorrhizal pot trials

Both inoculated and control plants were healthy after six months. The shoot number of inoculated plants was greater than un-inoculated plants (Fig. 2) with an ANOVA P value of 0.07.

Table 1. Mycorrhizal associations of *Borya mirabilis* over one year, showing average infection of 30 roots from each season and the type of arbuscular mycorrhizal infection

Season	Mean total % infection	Mean % infection AM	Mean % infection EM	AM structures
Summer	0.8 ± 0.24	0.43	0.37	Spores
Autumn	7.8 ± 0.88	4.6	3.2	Spores and coils
Winter	17.6 ± 0.87	14.9	2.7	Coils
Spring	12.4 ± 0.92	12	0.4	Coils

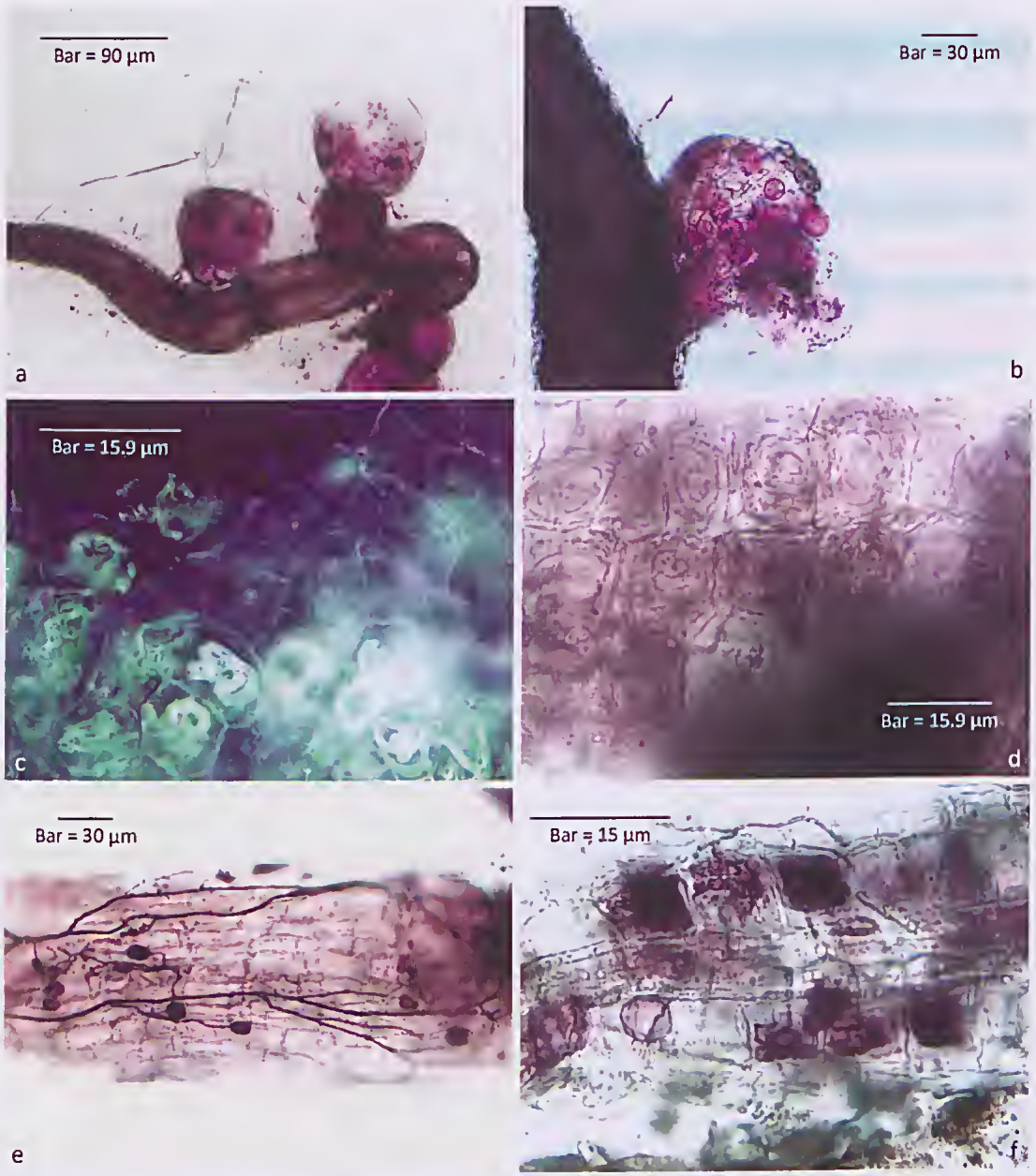

Figure 1. a. Low-power light microscope image of *Borya mirabilis* lateral root with nodular offshoots, showing indeterminate growth; **b.** *B. mirabilis* root sampled in summer, with nodule containing spores consistent with endogone-type mycorrhizal infection; **c.** *B. mirabilis* root sampled in winter with nodule containing endogone-type arbuscular mycorrhizal coils; **d.** Paris-type arbuscular mycorrhizae in *Callitris rhomboidea* roots collected from *B. mirabilis* field site; **e.** arbuscular mycorrhizae in roots of *Acacia stricta* collected from from *B. mirabilis* field site; **f.** arbuscular mycorrhizae in *Themeda triandra* roots collected from *B. mirabilis* field site

Associated species mycorrhizal examination

Of the 19 associated species from the *B. mirabilis* field site, 12 had AM (Table 2). Of these, *Callitris rhomboidea* (Fig. 1) displayed the most morphologically similar mycorrhizal association to that observed for *B. mirabilis*, with a Paris-type association. The other plants that displayed AM in the majority of slides examined were *Acocio stricta* (Andrews) Willd. (Fig. 1), *Dodonaeo viscosa* (L.) Jacq., *Kunzea parvifolia* Schauer, *Leptospermum scoporium* J.R.Forst & G.Forst and *Themeda triandra* Forrsk. (Fig. 1). *Grevillea aquifolium* Lindl. had proteoid roots, but *G. alpino* Lindl. had AM and EM.

Discussion

We have demonstrated for the first time that *Borya mirabilis* has a mycorrhizal association and that it is typical of the nodular AM (Endogone type) association. While EM associations were found, they were in low numbers, particularly over spring when the *Borya* plants were actively growing (Table 1). Nodular formations on

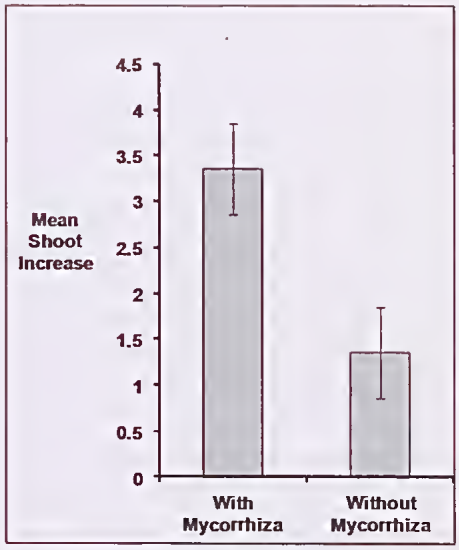

Figure 2. Mean shoot increase over six months of *Boryo mirobilis* plants inoculated with mycorrhizal roots and those without. Bars refer to 2x standard error of the mean of each treatment

Table 2. The presence of different types of mycorrhizae or proteoid roots in plant species associated with *Boryo mirobilis* in the field; the dominant type shown as ++

Species	EM	AM	Ericoid	None	Orchid	Proteoid Root
Acocio stricto	+	++				
Rytidospermo setoceum				++		
Collitris rhomboideo		++				
Colytrix tetrogono	+	+				
Dodonoeo viscoso	+	++				
Drosero oberrons				++		
Eucolyptus olaticoulis	++	+				
Gonocorpus mezionus	+	+				
Grevilleo oquifolium						++
Grevilleo olpino	++	+				
Kunzeo porvifolio	+	++				
Lepidosperma viscidum				++		
Leptospermum scoporium	+	++				
Leucopogon virgotus			++			
Meloleuco decussoto	+					
Phyllonthus hirtellus	++	+				
Thelymitro ixioides					++	
Themedo triondro		++				
Thryptomene colycino	++	+				

Present on site but not examined: *Centrolepis oristoto*, *Centrolepis strigoso*, *Drosero peltato* subsp. *ouriculata*, *Austrostipa hemipogon*.

Boryo roots or the AM association residing inside have not been reported previously.

Arbuscular mycorrhizal fungi, usually belonging to the Glomales, are associated with plant roots forming mycorrhizal nodules (Huguenin 1969). These nodule-inhabiting mycorrhizae are associated with many species in the Podocarpaceae (Saxton 1930; Morrison 1963), Araucariaceae, Phyllocladaceae and Casuarinaceae and the Caesalpiniodeae in the legumes (Béreau & Garbaye 1994). This type of AM mycorrhiza is characterised by the cortex of the root being colonised, but without any association in the endodermis. The cortex is also surrounded by a periderm that contains several layers of dead cells (Duhoux *et al.* 2001), unlike normal AM associations, which only infect unsuberised roots (Duhoux *et al.* 2001).

The nodular roots of *B. mirabilis* do not have determinate development, unlike most nodular mycorrhizal associations (Duhoux *et al.* 2001). In *B. mirobilis* a new nodule can form from the tip of old nodules, leading to an elongated moniliform nodular lateral root. *Gymnostoma nodiflorum* (Thunb.) L.A.S.Johnson (Casuarinaceae) is one of the few plant species with nodular mycorrhizal roots that also has the ability to reactivate the apical meristem to extend its lateral roots (Duhoux *et al.* 2001).

This is the first report of seasonal changes in the AM structures associated with *Boryo* species or nodulated AM species. It would therefore be interesting to see if these same seasonal changes occur in other *Boryo* species and if the morphological seasonal changes still occurred if plants were not allowed to go through their desiccation-tolerant phase in summer, in *ex-situ* conditions.

The addition of nodule-inhabiting AM fungi from the field site of *B. mirabilis* to the *ex-situ* population improved plant growth. Benefits of *Endogone*-type (nodule-inhabiting) AM, including increased growth, health and nutrient uptake, have been documented previously (Baylis 1959).

Arbuscular mycorrhizae cannot currently be cultured asymbiotically. They require host roots to remain viable. This poses difficulties for developing plans to enhance the recovery of the *B. mirobilis* population, not only in the *ex-situ* collection, but also in the reintroduction sites. For the *ex-situ* population, it is suggested that the mycorrhizal fungi from the field be introduced and kept in labelled pots, and that roots from these plants be used to inoculate cuttings as they become available. In this way, plants translocated to external sites could be inoculated with beneficial mycorrhizal fungi that will presumably aid their establishment and growth. The collection of endangered plant roots, especially from a field site that is infected with *Phytophthora cinnamomi*, is not a viable means of introducing mycorrhizal fungi to *ex-situ* populations.

The most similar mycorrhizae among the associated species was that of *Callitris rhomboidea*. The AM of *C. rhomboidea* have not been described previously. However, Pattinson *et al.* (2004) found that the species produced AM when inoculated with arbuscular mycorrhizal fungi. Other *Collitris* species are well known for forming AM. In particular, *Collitris glaucophyllo* Joy Thomps. & L.A.S.Johnson has AM that are morphologically similar to that found on *C. rhomboideo* (Dickson *et al.* 2007). Presence of AM in *C. rhomboideo* means that it could be used as a source of inoculum for pots and as an indicator of suitable translocation sites for *B. mirabilis*, although trials are needed with *ex-situ* plants to see if cross-innoculation or translocation would be beneficial.

The majority of mycorrhizal associations among associated plants from the *B. mirabilis* field site were consistent with reports of known mycorrhizal associations (Brundrett 2008). There were, however, several unexpected results. In particular, *Grevilleo olpino* (Proteaceae), which would be expected to have proteoid roots and thus no mycorrhiza, was found to have an EM and AM associations. However, it is not alone in the Proteaceae in having an AM association; these associations have also been recorded in *Bonksio ericifolia* L.f. and *Telopeo speciosissima* (Sm.) R.Br. (Wang & Qiu 2006).

Future work on the nodular mycorrhizal associations of *B. mirobilis* should more precisely determine the structure of the AM fungi inside the nodules and should also attempt to identify the fungi responsible by DNA analysis. Such analysis will require careful washing and removal of the nodules before extraction, preferably with fresh material, which may increase the initial concentration of fungi in the extract compared to plant DNA. Amplification of Glomalean AM fungi has already

been successfully achieved from nodular mycorrhiza of *Gymnostoma* (Duhoux *et al.* 2001).

With the aid of molecular identification, the fungi in the AM that was associated with the species in the field and was beneficial in the *ex-situ* collection could be identified. Further research could then test to see if the same fungus is present in mycorrhizae of other associated species that have been shown to form AM associations, in particular *Callitris rhomboidea*. If this is the case, then the roots of these non-endangered species could be used for further fresh inoculations of the mycorrhiza from the existing field site to the *ex-situ* population.

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